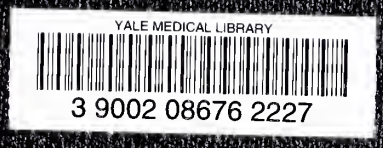

IN VITRO STIMULATION OF HUMAN UMBILICAL CORD BLOOD LYMPHOCYTES BY ULTRAVIOLET-IRRADIATED HERPES SIMPLEX VIRUS

THOMAS R. MOORE

1979

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IN VITRO STIMULATION OF HUMAN UMBILICAL CORD BLOOD LYMPHOCYTES BY ULTRAVIOLET-IRRADIATED HERPES SIMPLEX VIRUS

THOMAS R. MOORE '79

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DEDICATION

To Dr. George Miller MD whose interest, enthusiasm and dedication to the things of science and the life of the soul have made this project and my association with him satisfying, enjoyable and even inspiring.

HERPESVIRUSES AND ONCOGENESIS

I. Epidemiological and *In Vivo* Evidence.

The concept that herpesviruses are intimately associated with, if not indeed etiologic agents of, some forms of human cancer is scarcely two decades old. The demonstration in 1964 by Epstein et al.[1] that a herpesvirus could be found in cultured lymphoblasts of African Burkitt lymphoma was highly suggestive.

Work with the Lucke carcinoma of the frog earlier established the presence of herpesvirus particles in the tumor cells[2], but it was not until 1969 that Mizell et al. were able to induce tumors *in vitro* in frog tissue using the Lucke virus.[3] This work was later confirmed *in vivo* by Naegle et al.[4]

Similarly, Marek's tumor of chickens, long known to be a contagious lymphoma, was found to harbor a herpesvirus in 1967.[5] The discovery that immunizing chickens with a related herpesvirus of turkeys (1970) could prevent tumor formation[6] added compelling evidence that herpesviruses may be oncogenic in animals. Efforts to develop more direct associations for herpesviruses and malignancies in mammals ensued.

In primates, studies established that Herpes saimiri, a non-pathogenic herpesvirus in its natural squirrel monkey

host, induced lymphomas in owl monkeys and cottontop marmosets[7] and fatal leukemias in other sub-human primates.[8] In 1972, another simian herpesvirus, Herpes ateles, was shown to be oncogenic in South American monkeys.[7,9] These investigations have not only provided considerable evidence of herpesvirus oncogenicity in primates but also supported a concept which evolved from the Lucke virus studies:[4] that a latent, benign viral infection in one setting might result in malignant tumor formation in another.

Efforts to demonstrate oncogenesis by human herpesviruses have been nearly as fruitful. Epidemiological association of Epstein-Barr virus (EBV) with Burkitt's lymphoma was provided by Henle's report of the correlation of high EBV antibody titers in lymphoma patients; geometric mean titer in the tumor patients was 275 while control patients' titers averaged 37.[10] Subsequently, EB virus was cultured from many lymphoblastoid cell lines derived from Burkitt lymphoma tissue;[11,12] EBV antigens[13] and EBV specific DNA sequences were identified[14-17] in the nuclei of Burkitt lymphoma cells. Similar antigenic associations[18,19] and nucleic acid homologies[19,20] have been described in the tissues of nasopharyngeal carcinoma.

More direct evidence of oncogenicity was obtained by inoculation of EBV into sub-human primates. Malignant lymphomas produced by EBV in cottontop marmosets[21] and owl monkeys[22,23] yielded EBV-producing cell lines <u>in vitro</u>;

and EB nuclear antigen[24] and EBV genomes[25] were detected in the primary tumor cells as well.

Implicating other herpesviruses in human malignancy has been less rewarding. To date, there is no *in vivo* evidence linking cytomegalovirus (CMV) or varicella zoster virus (VZV) with oncogenesis[26] although Albrecht et al.[27] induced *in vitro* malignant transformation of hamster cells with ultraviolet-irradiated CMV. Studies with Herpes simplex virus (HSV) have been more suggestive, and are recently well reviewed.[26,28-30]

Naib[31] first suggested the association of HSV type 2 with cancer of the uterine cervix. Nahmias et al. found that the incidence of cervical dysplasia and *in situ* carcinoma was increased 2 to 8-fold among HSV type 2 seropositive individuals compared to seronegative controls,[32] strengthening Gagnon's observation some fifteen years earlier that not a single case of cancer of the cervix was found among 13,000 Catholic nuns over a 20 year survey period.[33]

Attempts to identify HSV-2 specific antigens in cervical tumor tissue have been generally unsuccessful.[34] Aurelian was able to detect immunity to an HSV-2-induced tumor-specific antigen in cervical cancer patients while no such antibody was found among controls.[35] Several investigators reported finding HSV antigens in 2-20% of exfoliated cells in 60% of Papanicolau smears of patients with invasive cancer, versus 0-6% in control smears.[34,36]

Evidence documenting HSV DNA in tumor cells has been contradictory,[37,38] although Frenkel et al. detected a fragment of Herpes simplex type 2 DNA in the cells of a cervical carcinoma;[39] Aurelian et al. (1971) reported recovery of HSV-2 from cervical tumor cells in tissue culture[40] and complete virus particles were observed in cells of 2 of 14 cervical tumors by Herrera et al.[41]

Compared to the weight of data supporting the role of EBV in oncogenesis, the evidence relating HSV to human cervical cancer is highly suggestive, but by no means conclusive.[27,30,42]

II. Herpesvirus Infection: Lytic and Latent Mechanisms.

Interpretation of the epidemiological and biological associations of herpesviruses and malignancy depends on understanding the dynamics of cell-virus interaction during infection. For example, a high proportion of individuals with cervical cancer have serological and histologic history of HSV-2 infection, yet it is clear that oncogenic transformation of infected cells never occurs in the majority of affected persons.[43] Similarly, Epstein-Barr virus infection is extremely prevalent world-wide[44] but only a tiny fraction can be associated with Burkitt's lymphoma or nasopharyngeal carcinoma.[45] Is there, then, more than one way in which the infecting virus influences the cell?

It is likely that all herpesviruses are capable of establishing both lytic[46] and latent[47] infections and that the specific mode of infection depends to a considerable degree on the native characteristics of the host cell. In the case of Epstein-Barr virus, human umbilical cord lymphocytes transformed by EBV into cell lines with the ability to grow continuously in culture produce no detectable virus despite the presence of EB nuclear antigen (EBNA) and EBV DNA in the transformants.[48] A non-productive lymphoblastoid line (Raji) with similar characteristics has been isolated from Burkitt lymphoma.[49] Infection of marmoset lymphocytes with the same strain of EBV results in transformation and considerable virus production.[48]

Epithelial cells are characteristically permissive for the replication of Herpes simplex virus: the outcome is cell lysis and death.[38,46] Other cells, e.g. those of neural origin, appear to be non-permissive for replication and the usual outcome of HSV infection is latency.[49]

Stevens has summarized the data on this point.[47,50] Intact virus and hybridizable DNA sequences of latent HSV have been demonstrated in trigeminal ganglion neurons after experimental chronic herpetic eye infection in rabbits[51] and mice;[52] reactivation of dormant infection has been achieved by neurectomy[53] or imposition of septic shock.[50] Baringer[54] documented recovery of infectious HSV-1 from 31 of 71 human cadaver trigeminal ganglia examined, and HSV-2

from 5 of 46 sacral ganglia, suggesting that 50% of the human population may harbor latent HSV in their sensory ganglia.[55,56]

The ability of HSV to establish latent infection in human cells is now well-documented but it is less certain whether HSV may be capable of oncogenic transformation in human disease. The demonstration of HSV DNA and HSV antigens in cervical carcinoma tissue has bolstered speculation that HSV may also be a "transforming virus" in the production of human cervical cancer.[57] Attempts to support this thesis with *in vitro* techniques are discussed below.

III. Oncogenicity and Herpesviruses: *In Vitro* Evidence.

Insights from *in vivo* and epidemiological studies led to experiments combining cells and herpesviruses *in vitro* with hopes of inducing oncogenic transformation under controlled circumstances.

A. Transformation by Epstein-Barr Virus.

The first report by Henle et al.[11] in 1967 of transformation by EBV of human leukocytes co-cultivated with X-irradiated cells from a Burkitt's lymphoma tumor was followed shortly by Pope et al. (1968) who transformed

fetal leukocytes using the filtered extracts of an EBV-producing leukemia cell line.[12] This work was confirmed by Miller et al. in 1969.[58]

In these and the numerous subsequent reports, the *in vitro* transformation of leukocytes by EBV exhibits the following characteristics:

1) EBV from several sources produces transformation, including Burkitt cells,[11] leukemia,[12] infectious mononucleosis[59] and previously transformed monkey leukocytes.[48]

2) a single target cell--probably a "B" lymphocyte[60]--can be derived from several sources, including fetal bone marrow[61], umbilical cord blood[52] and peripheral adult blood.[58]

3) morphologically and biologically, the transformed cells are lymphoblasts[63] which are capable of surviving and dividing continuously in culture.

4) the EBV DNA is associated with the host cell's genome and can be detected by the presence of viral antigens (EBNA)[13] and hybridizable DNA sequences.[14]

Certain aspects of the *in vitro* EBV transformed cell are strikingly similar to those of the Burkitt lymphoma cell, i.e. the presence of EBV DNA and antigens in lymphoid cells with altered growth characteristics. On the strength of these similarities, the EBV model of oncogenesis is a convenient standard by which other "transformation" systems can be measured.

B. Transformation by Herpes Simplex Virus.

Efforts to induce *in vitro* transformation by other human herpesviruses are in general frustrated by the tendancy of the virus to undergo lytic infection in the host, resulting in cell death before transformation.[64] A number of experimental techniques have been brought to bear on this problem:

1) development of temperature-sensitive mutant viruses (Ts) which are infectious at physiological (permissive) temperatures but able to infect cells without lytic reproduction at elevated (non-permissive) temperatures.[65-69]

2) partial inactivation of virus by pre-treatment with sub-lethal doses of ultraviolet (UV) light,[70-72] chemicals[73-76] or light-activated chemicals.[72,77]

3) suppression of viral reproduction after infection by maintaining infected cell cultures at elevated temperatures.[78]

4) use of specific viral DNA fragments defective for lytic functions to "transfect" cells directly.[79,80]

The thesis behind these maneuvers is that if a portion of the herpesvirus genome which directs viral reproduction and cell lysis can be suppressed, deleted or bypassed, oncogenic functions of the virus may be unmasked.[81]

Owing to the lytic behavior of HSV in epithelial tissues, transformation attempts were begun with partially-

inactivated virus, using a variety of non-lymphoid cell types as targets. These studies are summarized in Table I.

Using ultraviolet-irradiated HSV, in which infectivity had been reduced by 10^{-4}, Duff and Rapp[38,82,83] were able to transform hamster embryo cells at a frequency of 10^{-5} transformants per normal cell. The transformants had the following properties:

1) transformation was observed with a minority of virus strains tested (2 of 12);[35]

2) transformants grew out into stable tissue culture cell lines;[38,68,72,84,85]

3) transformed lines expressed HSV surface antigens;[38,86]

4) transformed cells induced tumors in Syrian hamsters on inoculation;[38,68,82]

5) transformants were morphologically poorly differentiated.[80,82]

It should be noted that the transformed lines yielded no infectious virus or cytopathic effect in culture with susceptible cells.

HSV DNA fragments in these transformants have been reported to represent 10%[87] and 8-32%[88] of the genome, although Summers[37] could find no sequences in seven strains of HSV-transformed cell lines. Morphological and tumorigenic transformation of rodent cells was also achieved by Duff, Rapp et al. using photodynamic inactivation of

TABLE I

TRANSFORMATION OF NON-LYMPHOID CELLS BY HSV

Cell Type	Virus Inactivator	Outcome: Cell Lines	Outcome: Tumors	TK^+	Reference
Hamster Embryo Fibroblast	UV (5'-12' exposure)	+	+		38,67,82 83,102
	Temperature-Sensitive Mutant (Ts)	+	+		67
	Chemical	+			74
	Photodynamic	+	+		77
Rat Embryo Fibroblast	Ts	+	+		68
Mouse Fibroblast	UV (10'-12' exposure)	+	+	+	85,103
	DNA Fragments			+	79
Human Embryo Lung	Cell Temperature = 42^oC	+			78

HSV.[73,78,89]

Biochemical transformation, in which the transformants are morphologically unchanged but exhibit altered enzymatic characteristics, has been accomplished with HSV in mouse "L" cells. Culturing infected cells in medium lethal for the absence of thymidine kinase activity[85] selects transformants possessing this enzyme (TK^+) while uninfected or untransformed cells lacking the enzyme (TK^-) do not survive. Biochemical transformation of rodent cells lacking thymidine kinase was induced with ultraviolet-irradiated HSV (UV-HSV),[85] with live HSV in non-permissive Rous-sarcoma transformed rat cells[90] and more recently with specific HSV DNA fragments alone.[80] In subsequent work, hybridization techniques have detected 6 copies of HSV DNA fragments representing 15% of the viral genome in the TK^+ transformed cells, with none reported in the TK^- cells.[91] The newly induced enzyme is considered to be of viral origin, in that the transformed cell enzyme's substrate specificity and electrophoretic mobility are identical to the native HSV enzyme.

IV. Interaction of Lymphoid Cells and Herpes Simplex Virus.

Interest in transforming lymphoid cells with HSV grew out of the experience with EBV, although *in vivo* evidence that HSV infects lymphocytes latently or lytically is lacking.

A. Replication.

Experiments demonstrating the extent to which HSV can infect and replicate in lymphoid cells are summarized in Table II. It will be readily recognized that in contrast to epithelial tissues, primary unstimulated lymphocytes support HSV replication very poorly.[89,95-97] However, lymphocytes stimulated with mitogens (phytohemaglutinin (PHA), concanavalin A (Con A), antilymphocyte globulin) support HSV replication over a short (2 to 5 day) period before the lytic effects of the virus presumably kill the culture.[95,96-99] The virus reportedly grows well in both T and B lymphocyte cell lines[97,99,100] and the initial belief that only T cells replicate virus[101] appears now to be unfounded.[97,99]

Why lymphocytes are especially resistant to productive infection by HSV is not clear. The fact that cells actively synthesizing DNA (mitogen stimulated or cell lines) support replication much more readily suggests that the state of cellular metabolism may play an important part.[96] Of interest is the report by Daniels et al.[95] of HSV replication in human macrophages <u>in vitro</u>. Attempts to infect freshly isolated macrophages resulted only in "abortive infection" in which no infectious virus was produced but empty viral capsids and HSV antigens were detected in the cells. In fresh cultures only $1/10^5$ cells was productively infected,

TABLE II

REPLICATION OF HERPES SIMPLEX VIRUS IN HUMAN LYMPHOCYTES

Cell Type	Replication ± Mitogen (+)	Mitogen (-)	Peak Titer/Culture Interval	Reference
Adult Peripheral Lymphocytes:				
Mixed	+	-	2-3 days/ 10 days	96-99,104
B-Enriched	+	+ (3/5 Donors)	3 days/ 7 days	95,97
T-Enriched	+	- (5/5 Donors)	" "	95,97
Umbilical Cord Lymphocytes, Mixed		± (4/8 Donors)	5 days/ 5 days	105
Cell Lines:				
T-Cell		+	3 days/ increasing	97,99,100
B-Cell		+	" "	97,99,100

but in cultures "aged" *in vitro* for 3 days, susceptible cells increased to 1% and rising viral titer was noted over the next four days. Confirmation of this work or elucidation of the fate of the abortively infected cells has not been reported.

B. Antigenic Stimulation.

Efforts to induce transformed lymphocyte cell lines with HSV have been unsuccessful to date, but several investigators have succeeded in transient stimulation of lymphocytes pre-immunized to HSV *in vivo*. These experiments are summarized in Table III.

Rosenberg's work[106-108] with lymphocytes of HSV-immune rabbits documented that significant stimulation (evidenced by increased uptake of tritiated thymidine (^{3}HTdr)), occurred 3 days after infection with HSV, an effect which was increased by UV treatment of the virus. The stimulation was transitory (3 to 5 days) and was associated with increased synthesis of cellular, not viral DNA.[107] No stimulation occurred in cells from antibody negative animals.

Similar results were achieved with both live and inactivated HSV in lymphocytes of immune human donors.[75,76, 109-112] Corey et al.,[110] and Lopez et al.[111] have studied the stimulation of lymphocytes from persons with active or recurrent herpes labialis infection by UV irradiated HSV in

some detail. They found stimulation indices (= ratio of ^{3}HTdr uptake by HSV-infected lymphocytes compared with the uptake by uninfected controls) as high as 69 in patients with symptomatic herpes infection. The magnitude of lymphocyte stimulation correlated with the stage of virus infection: the peak of lymphocyte stimulation index was observed to coincide with the maximal rate of virus shedding.[110] Stimulation decreased to lower levels in convalescent, but antibody positive individuals.[110-111]

Corey observed that cells from neutralizing-antibody negative donors produced stimulation indices of 0.8 $\pm$ 0.2. This finding was confirmed by Starr[109] and Rasmussen[113] and by Shillitoe et al.[75,76] who also noted that two seronegative control patients whose stimulation index was unexpectedly greater than 2.0, developed active vesicular disease and seroconversion within two weeks.

The latter observation may help explain the divergent finding by Lopez et al.[111] of an average stimulation index of 4.5 in 11 asymptomatic, seronegative controls but nevertheless, this result is clearly at odds with those of the other studies cited.

The bulk of the data suggest, then, that UV-irradiated HSV is antigenic to lymphocytes in immune humans and rabbits but the resulting stimulation is transitory and probably linked to a cellular immune response to antigens on the virion rather than mediated by the viral genome. The in-

TABLE III

ANTIGENIC STIMULATION OF LYMPHOCYTES BY HERPES SIMPLEX VIRUS IN VITRO.

Cell Type	Antigen	Stimulation Index* Donor Antibody (+)	Antibody (-)	Reference
Rabbit Spleen	HSV + UV	9.4	0.45	106-108
	Live HSV	4.3	0.03	
Human Peripheral:				
Mixed	HSV + UV	69	4.5	111
		17	0.8	116
	HSV + Heat	1.4-1.8	0.5-1.0	109,113
	HSV "Antigens"	6-12	2-5	75,76,112
B-Enriched	Live HSV	2.0	1.0	75,76
T-Enriched	Live HSV	7.5	1.0	75,76

*Stimulation Index = $\frac{^{3}\text{HTdr uptake by infected cells}}{^{3}\text{HTdr uptake by uninfected cells}}$

creased ^{3}HTdr uptake is due to increased cellular, not viral DNA synthesis, as evidenced by cesium chloride gradient DNA analysis[107] and the finding that live HSV infected cultures do not exhibit increased thymidine uptake despite ongoing viral synthesis.[97]

C. Mitogenic Stimulation.

Mitogenic stimulation is distinguished from antigenic stimulation by the ability of the mitogen to cause stimulation of both immune and non-immune cells, whereas antigens stimulate only pre-immunized cells.[114] Demonstration of stimulation of non-immune lymphocytes by HSV would be a first step toward the goal of oncogenic transformation.

Studies of non-immune lymphocytes and HSV have been few. Review of the experiments cited in Table III shows that stimulation of lymphocytes from HSV-antibody negative humans is generally unsuccessful. In the cases where stimulation was reported,[111,112] interpretation is confounded by the possibility of cross-reaction of lymphocytes immunized to related antigens.

Experiments with lymphocytes derived from the spleens of nude mice conducted in separate laboratories[115,116] have documented that live HSV is strongly mitogenic in mice (Table IV). Moreover, the stimulation is maximized in "B" enriched cultures and abolished by heat, UV or antiserum

TABLE IV

MITOGENIC STIMULATION OF LYMPHOCYTES BY HERPES SIMPLEX VIRUS IN VITRO.

Cell Type	Mitogen	Stimulation Index*	Remark	Reference
Mouse (Nu-Nu) Spleen:				
Mixed	Live HSV	10-50	1) SI 1.0 for HSV + UV, heat, antiserum	115,116
B-Enriched	Live HSV	20-50		
T-Enriched	Live HSV	1.0	2) No infectious virus production.	
			3) SI Maximum @ 2-3 days	
			4) 6/13 strains of HSV were mitogenic	
			5) Cellular DNA stimulated, not viral.	
			6) Macrophage depletion required for stimulation	
Human Umbilical Cord:				
Mixed	HSV + Heat	1.0		109

*Stimulation Index (SI) = $\frac{^{3}\text{HTdr uptake by infected cells}}{^{3}\text{HTdr uptake by uninfected cells}}$

inactivation of the virus. Only a minority of the virus strains tested[116] possessed stimulatory activity, however.

These results are in contrast to the "antigen" stimulation studies cited in Table III: the effect requires live virus, and maximal stimulation occurs at 3 days versus 7 days in the antigen studies.[110,111]

Investigation of lymphocyte response to HSV in immune-deficient/compromised individuals has not been reported but one experiment involving heat-inactivated HSV and human umbilical cord lymphocytes found no stimulation.[109]

V. Modes of Latent Infection and Transformation.

Transformation of cells by both Epstein-Barr virus and Herpes simplex virus is well-documented, although the inability to recover infectious virus from any HSV transformants[82] and to demonstrate viral genome sequences in all HSV-induced cell lines[38] attests to clear differences between HSV-induced and EBV-induced transformation. When detectable, HSV DNA in transformed cell lines is only a small fragment, in contrast to the multiple copies of complete genomes found in EBV lymphoblastoid lines. Intact EBV transforms cells but HSV must be partially inactivated to perform this function. The striking similarity in the UV doses used to achieve maximum transforming efficiency

(10-15,000 ergs/mm^2-sec) for both tumorigenic and biochemical transformation may be indicative of the target sizes of the "lytic" and "transforming" parts of the HSV genome, but no such differentiation of functions has been clearly demonstrated in EBV.

Regarding the setting in which latent herpesvirus infection may occur, four prominent possibilities have been proposed:

1) viral genome is present in cells but unexpressed; it is capable of reactivation into a lytic, productive cycle,[47,53] e.g. recurrent herpes labialis, h. progenitalis, h. zoster.

2) viral genome, or part of it, is present and expressed as oncogenic transformation of the host cell, with malignancy as the outcome; lytic functions of the genome are either missing or suppressed[16,17] e.g. EBV in Burkitt lymphoma, Marek's virus and chicken lymphoma.

3) viral genome is present during the initial transformation of the cell, but continued presence of the genome is not required for ongoing reproduction of the transformant progeny e.g. the so-called "hit and run" model of carcinogenesis.[92]

4) viral genome or fragment is present in the infected cell as the inducer/promotor of a latent C-type RNA virus which in turn transforms the cell.[93,94] The studies cited in Table I provide support for each of the first three

proposals although the evidence is inconclusive for all. The last possiblity, that of oncornavirus activation, was suggested by the finding of Hampar et al.[94] that infection of rodent cells with UV-HSV resulted in the induction of a xenotropic C-type virus. Kraiselburd et al.[93] extended these observations by noting that the optimum UV dose for xenotropic virus induction closely parallelled that for maximum thymidine kinase transformation and proposing that the genes of HSV causing this effect represent a small fraction of the genome. HSV DNA synthesis was not required for activation of the C-type virus.[93]

Although it may be helpful to conceptualize transformation by herpesviruses occurring in latent infection as occurring by one or more of the mechanisms cited above, it is not at all clear which, if any, of these is pertinent to transformation by Herpes simplex virus. Further studies are required to elucidate the details of HSV transformation.

VI. Rationale for Experiments with Herpes Simplex Virus and Non-Immune Lymphocytes.

The purpose of the experiments presented in this paper was to investigate the possiblity that primary lymphocytes could be stimulated or transformed by UV-irradiated HSV.

Choice of the lymphocyte was based on several consid-

erations. First, lymphocytes had been shown to be relatively non-permissive for HSV replication.[96-99,109] Successful stimulation and transformation experiments in the past have been conducted in non-permissive cells (cf. umbilical cord lymphocyte with EBV[62] and the mouse spleen lymphocyte and HSV[115,116]). Further, the existence of lymphoblastoid cell lines derived from cancer patients in which no virus can be identified suggested that perhaps HSV could be a "transforming virus" for lymphocytes. Use of non-immune, washed lymphocytes collected from umbilical cord blood at the time of fetal delivery would minimize confusion about lymphocyte-antigen cross reaction or mitogenesis associated with antigen-antibody complexes.[114]

Similarly, partial inactivation of the virus was considered likely to maximize stimulatory properties of the virus since lymphocytes had been shown to support limited replication of HSV over the time period in which stimulation was expected to be evident (3 to 7 days).[109] Choice of ultraviolet irradiation was based on the documented ability of UV-irradiated HSV to transform rodent cells,[38,40] and in accordance with the hypothesis that the lytic functions of the HSV genome have a larger UV target size than the transforming functions.[70,82]

Assays for stimulation of DNA synthesis, manifested by the level of thymidine uptake, determinations of the

infectivity of live and inactivated virus, and attempts to induce transformation of cells in different conditions of tissue culture were conducted.

MATERIALS AND METHODS

Virus. HSV type 1, strain No. 15077, was derived from a brain specimen obtained from a patient with encephalitis. It was isolated in the Vero line of African Green Monkey cells and subsequently passaged three times in the AH-1 Green Monkey Kidney (GMK) cell line. The virus stock titer was 5 X 10^5 plaque-forming units (pfu)/0.1 ml in Vero cells.

Prior to use the virus was passaged once more in GMK AH-1 cells at a multiplicity of infection of 1 pfu/cell. After three or four days, the supernatant fluid was collected and the cellular debris removed by low-speed centrifugation. The titer of this stock, designated "unpurified virus" was 10^6 pfu/0.1 ml.

"Purified virus" was subjected to the following further processing: the supernatant was centrifuged in a Beckman Type 19 rotor for 3 hours at 5°C, 20,000 rpm; the pellet was resuspended in 1 ml of Tris .05M and NaCl .15M buffer, layered on a 20-60% continuous sucrose gradient and centrifuged in an SW 27 rotor at 23,000 rpm for 90 minutes. A discrete, light-scattering band was collected and dialyzed against RPMI 1640 medium for 48 hours at 5°C. The virus was divided into 1.0 ml aliquots and stored at -70°C until use. "Purified virus" stock titer was 10^9 pfu per 0.1 ml. By electron microscopy[48] this stock consisted

of 82% enveloped virus particles, 18% capsids, and contained no cellular debris. All virus stocks were shown to be free of mycoplasma by culturing on mycoplasma agar.

UV Irradiation of Virus. Virus suspensions in growth medium (2 ml) were irradiated in 6 cm plastic culture dishes with a 25 watt GE Germicidal Lamp at a distance of 12 cm. Irradiation at this distance produced an ultraviolet light intensity of 25 ergs/mm^2-sec as determined by a dosimeter constructed and calibrated by R. Latarjet,[117] and kindly loaned by D. Rupp. During irradiation the dishes were kept on ice and rotated 100 revolutions per minute on a rotary platform shaker, while exposed to atmospheric oxygen.

Plaque Titration of Virus. Stock and UV-treated virus (0.1 ml), at various dilutions in growth medium, were inoculated onto confluent monolayers of GMK AH-1 cells in 6 cm plastic culture dishes. After one hour, 3 ml of 1.5% methylcellulose in growth medium was added and the dishes were incubated at 37°C in 5% CO_2 in air. After 3 days, the dishes were drained, fixed in absolute methanol for 5 minutes and stained by the method of Giemsa. Characteristic plaques were counted and titer was expressed as plaque-forming units per 0.1 ml.

Cell Isolation. Human umbilical cord leukocytes (HUCL) derived by the Ficoll-Hypaque method[118] were used.

To obtain cell populations depleted of T lymphocytes, mononuclear cells (5-10 X 10^6 leukocytes) were mixed with a 1% suspension of sheep erythrocytes pre-treated with 25 units/ml of neuraminidase (Vibrio cholerae, Calbiochem), centrifuged for 10 minutes at 200 g and held at room temperature for one hour.[119] The resulting mixture of rosetting cells (E rosettes) and nonrosetting cells was then layered over a second Ficoll-Hypaque gradient and centrifuged for 30 minutes at 400 g. The population at the interface then contained less than 5% of cells which formed E rosettes. This population,designated "B" enriched cells, was concentrated to 4 X 10^6 cells / ml for thymidine assays.

To obtain cell populations enriched for macrophages, freshly isolated human umbilical cord lymphocytes were suspended in growth medium and cultured flat in tissue culture flasks. Non-adherent cells were washed off three times during 7 days. The cells were harvested by washing in calcium-free medium several times[122], assayed for purity by demonstration of phagocytosis of EAC-IgG reagent-treated erythrocytes[123] and infected as described above. These cells consisted of greater than 98% phagocytosing cells.

Cell Culture. Before exposure to virus, some leukocyte populations were maintained for 12-24 hours at 4X10^6 cells

per ml in tissue culture flasks. This had the effect of removing most of the mononuclear phagocytic cells (population designated "macrophage depleted."[120]) Medium, RPMI 1640, was supplemented with penicillin (50 U/ml), streptomycin (50 ug/ml), amphotericin B (1 ug/ml) and various concentrations of heat-inactivated (56°C, 30 minutes) fetal calf serum. 0.1 ml aliquots of cells were placed in wells of microtiter plates (Costar) at $4X10^6$ cells/ml. 0.1 ml virus or medium was added as inoculum. All cells were cultured at 37°C in an atmosphere of 5% CO_2 and air.

Cell Counts. At the end of the culture interval, 2-4 wells of cells from each infected and sham-infected group were pooled and assayed for viability (Trypan Blue exclusion) and cell number (Coulter Counter).

Infectious Centers Assay. To determine the fraction of leukocytes in which viral replication occurred, varying numbers of infected cells were plated onto a monolayer of HSV-1 susceptible cells (GMK AH-1): leukocytes in growth medium were inoculated with HSV at a multiplicity of 10:1 pfu per cell; after one hour an equal volume of rabbit antiserum to HSV (titer 1:40, E. Grogan) was added; after an additional hour the cells were washed twice in Hank's Balanced Salt Solution (HBSS) and plated onto GMK cell monolayers. The plates were cultured for 3 days at 37°C

under a layer of 1.5% methylcellulose, following which they were drained, fixed in absolute methanol and stained with Giemsa. Plaques were counted and the results expressed as infected cells/total cells plated X 10^{-3}.

Lymphocyte Stimulation Assay. Incorporation of tritiated thymidine (^{3}HTdr) into acid-insoluble material was measured in infected and sham-infected cultures during the last four hours of incubation by the addition of 0.1 ml of ^{3}HTdr (specific activity 6.7 Ci/mM) to each well. The final concentration was 5 uCi/ml. The reaction was stopped by placing the microtiter plate on ice for 20 minutes.

Acid insoluble material was collected by pipetting the contents of each well onto a 2.5 cm membrane filter (Millipore Corp., type HA, 0.45 um) and washing each well with a small volume of HBSS to which 1.0 ml of trichloroacetic acid (w/vol) was added. The filters were then rinsed twice in 5 ml of water, dried and placed in vials with scintillation fluid (Econofluor, New England Nuclear). Radioactivity was determined in a liquid scintillation counter.[121] Separate experiments demonstrated that ^{3}HTdr added to cells and collected on filters without incubation resulted in counts per minute less than 10% of the values from incubated cultures.

Assays were done with 6 to 8 wells per group;average activity was expressed as the mean of counts per minute

per well of each group and the standard error of the mean of these groups was consistently less than 10% of the mean. Lymphocyte stimulation was expressed as "stimulation index", i.e., the mean counts/minute of infected cultures divided by mean counts/minute of sham/infected cultures. Statistical significance of differences was determined by the Student 't' test.

RESULTS

Infected Centers Assay. Previous studies established that HSV replication in lymphocytes is a limited, transient phenomenon,[96-99,109] but the fraction of cells productively infected in culture has not been determined. This can be approximated by calculation of the fraction of lymphocytes exposed to HSV which will induce a characteristic plaque after incubation on a monolayer of permissive cells such as GMK.

Table V shows that fewer than 1/1000 freshly isolated umbilical cord lymphocytes will support lytic viral replication. However, if these cells are maintained in culture for more than 5 days, the number of susceptible cells increases almost ten-fold.

To determine if one type of lymphocyte was more susceptible, populations enriched for "B" cells and macrophages were prepared and assayed for infectious centers. As shown in Table V, the number of infectious centers formed by B-enriched cells and macrophages were not significantly different from the mixed cultures. Tritiated thymidine incorporation in parallel cultures of mixed umbilical cord lymphocytes increased almost two-fold after 7 days of *in vitro* culture without virus.[121]

UV Inactivation of HSV-1. Results of titration of virus

subjected to UV irradiation are shown in Figure 1. It will be noted that infectivity is rapidly inactivated during the first minute of exposure but a relatively resistant region is encountered from 1 to 6 minutes; essentially complete inactivation is achieved by 20 minutes of exposure. These results are nearly identical to those obtained by other investigators.[82,124]

Stimulation of Umbilical Cord Lymphocytes by HSV. Preliminary thymidine uptake assays revealed a small but significant stimulation of lymphocytes infected by HSV irradiated for 6' to 10' (stimulation index = 1.15). This effect was apparent only on the third and fourth days of incubation. Increasing the multiplicity of infection from 1:1 to 100:1 pfu/cell, the use of purified virus and enriching the cells for B lymphocytes did not improve the stimulation achieved, although the effect was consistently observed in several experiments.

A number of attempts by other investigators to demonstrate induction of cellular DNA synthesis by virus have been successful only when the cells were "metabolically arrested." Yamanishi et al. demonstrated a five-fold stimulation of hamster cells exposed to a temperature-sensitive mutant HSV only when the serum concentration was less than 1%.[66] Human embryo fibroblasts exhibited a stimulation index of 9 with HSV-2 when maintained at 42°C

in 0.2% fetal calf serum.[73] St. Jeor et al. induced cellular DNA synthesis with live CMV (stimulation index = 9) when human embryonic lung cells were incubated in 0.2% fetal calf serum, but not in higher concentrations of serum.[125]

Umbilical cord lymphocytes maintained in culture medium supplemented with 20% fetal calf serum exhibit a marked spontaneous increase in ^{3}HTdr uptake during the first 7 days of culture.[121] This increase in background activity in uninfected cells may mask transient stimulatory phenomena which may be occurring simultaneously in the infected culture. Accordingly, stimulation assays were conducted in medium supplemented with 0.2% fetal calf serum; these are compared to parallel experiments in 20% fetal calf serum in Figure 3.

Significant stimulation ($p < .001$) in the 0.2% serum cultures was observed on the fourth day of incubation for all UV-treated virus; the highest values occurred with 6' and 10' UV doses (stimulation index = 1.7). Stimulation was significant ($p < .001$) in the 20% fetal calf serum cultures only for 10' UV-HSV infected cells, but the stimulation index was small (= 1.11). Thymidine uptake in cells exposed to unirradiated HSV was significantly depressed below control cultures in both serum concentrations tested.

Results similar to those of Figure 3 with mixed lymphocytes were repeated in five consecutive umbilical cord

blood specimens (mean stimulation index ± SEM = 1.58 ± 0.12).

Enrichment of lymphocytes for "B" cells significantly increased the stimulation index, as shown in Table VI. These cells had also been depleted of macrophages prior to inoculation. The values obtained for 0', 6' irradiated UV-HSV were unchanged, but 10' UV-HSV resulted in a stimulation index of 2.02, compared with 1.67 in mixed cells ($p < .001$).

Stimulation assays done on the seventh day after infection in 0.2% fetal calf serum resulted in a stimulation index (SI) of 1.43 ($p < .001$) for 10' UV-HSV and SI = 1.08 (p = non-significant) for 6' UV-HSV. Uptake assays for periods longer than 7 days have not been conducted in 0.2% fetal calf serum. Experiments with 20% serum at 14 days showed thymidine uptake less than 50% of controls for all virus preparations tested (0',6',10'). Continued stimulation of lymphocytes in reduced-serum medium at 7 days suggests that longer term activity of the UV-treated virus may be present.

To help exclude the possibility that the producer cells (GMK) elaborate a soluble mitogen which was responsible for the observed stimulation, both purified and unpurified virus suspensions were centrifuged for 1 hour at 23,000 rpm (SW 27 rotor), and the supernatant fluid was tested for stimulation. As shown in Table VI, centrifuged

supernatants of purified virus ($5X10^3$ pfu/0.1 ml) produced a stimulation index of 0.89 in B-enriched cells. Unpurified supernatant ($2X10^3$ pfu/0.1 ml) actually depressed thymidine uptake (stimulation index = 0.76) of B-enriched cells over control. Similar results were observed in mixed lymphocytes (Table VI). Stimulation assays in which lymphocytes were incubated with the supernatant culture fluid (SNF) removed from the producer cells (GMK) before inoculation with virus produced ^{3}HTdr uptake essentially identical to cultures incubated in RPMI 1640 medium alone (Figure 3).

Attempts to Isolate Transformed Cell Lines. Limited attempts to develop transformed cell lines from umbilical cord lymphocytes infected with UV-HSV were conducted in 20% fetal calf serum only. Although several of these cultures survived longer than 60 days, no difference was noted in survival time or morphology between infected and uninfected cultures. No transformed cell lines were isolated.

Cell Counts of Cultured Lymphocytes Infected with UV-HSV. It was important to determine if virus infection affected the survival of cells in culture over time, in order to interpret stimulation of lymphocytes after 4 days of incubation. Infection with HSV (multiplicity 100:1 pfu/cell) was conducted in fresh, mixed umbilical cord lymphocytes supplemented with 20% and 0.2% fetal calf serum. Cell

counts over days after infection, shown in Figure 3, were identical for all groups. Greater than 85% of the cells in each group were viable by Trypan Blue exclusion. Separate experiments with B-enriched populations indicate that the loss of 50-60% of the initial mixed inoculum of mixed cells in the first 1-2 days is due to the death of T lymphocytes, which do not survive well in the culturing methods used.

TABLE V.

Infected Centers in Lymphocyte Subpopulations Infected with HSV-1.

Infected Centers/10^3Cells

Cell Population	Pre-Infection Culture Interval	
	0-5 Days	6+ Days
Mixed Cells	0.6	4.8
B-Enriched Cells	---	8.0
Macrophages	---	6.0
Mean ^{3}HTdr uptake, Mixed Cells (counts per minute).	1956	3541

TABLE VI.

Comparison of Stimulation by UV-HSV:

Mixed vs "B" Enriched Leukocytes.

	Stimulation Index*		
	UV Time		
Cell Population	6'	10'	0' + Centrifugation**
Mixed Cells	1.60	1.67	1.03
"B" Enriched Cells	1.54	2.02	.89

* Stimulation Index = $\frac{^{3}\text{HTdr uptake, infected cells}}{^{3}\text{HTdr uptake, uninfected cells}}$

Assay on fourth day after infection of freshly isolated lymphocytes
Serum: 0.2% fetal calf serum
Multiplicity of infection: 1:1 pfu/cell

** UV-HSV centrifuged 20,000 (SW 27 rotor) for 1 hour.

FIGURE 1

INACTIVATION OF HSV-1 BY ULTRAVIOLET (UV) LIGHT

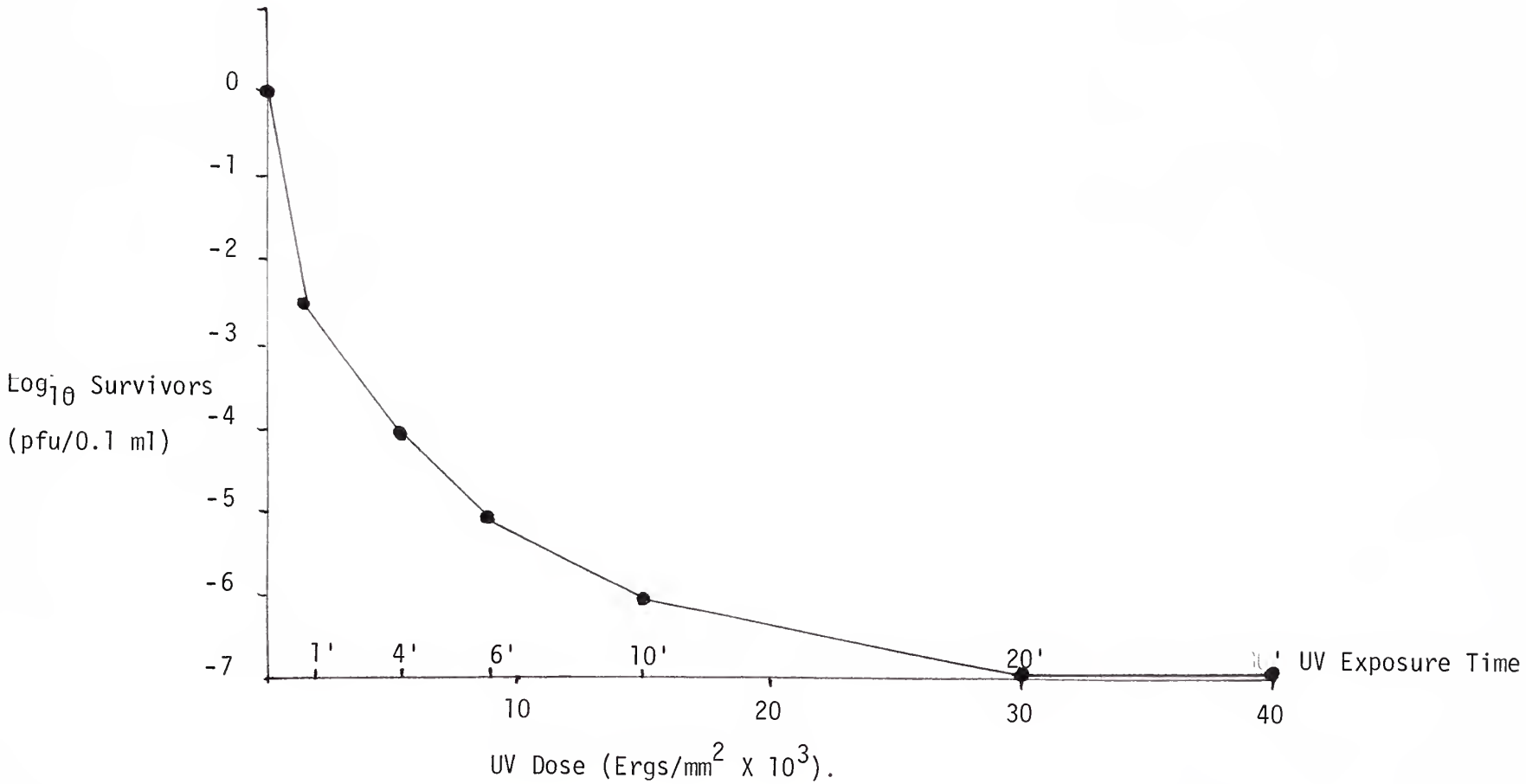


FIGURE 2

CELL COUNTS OF HSV-INFECTED LEUKOCYTES

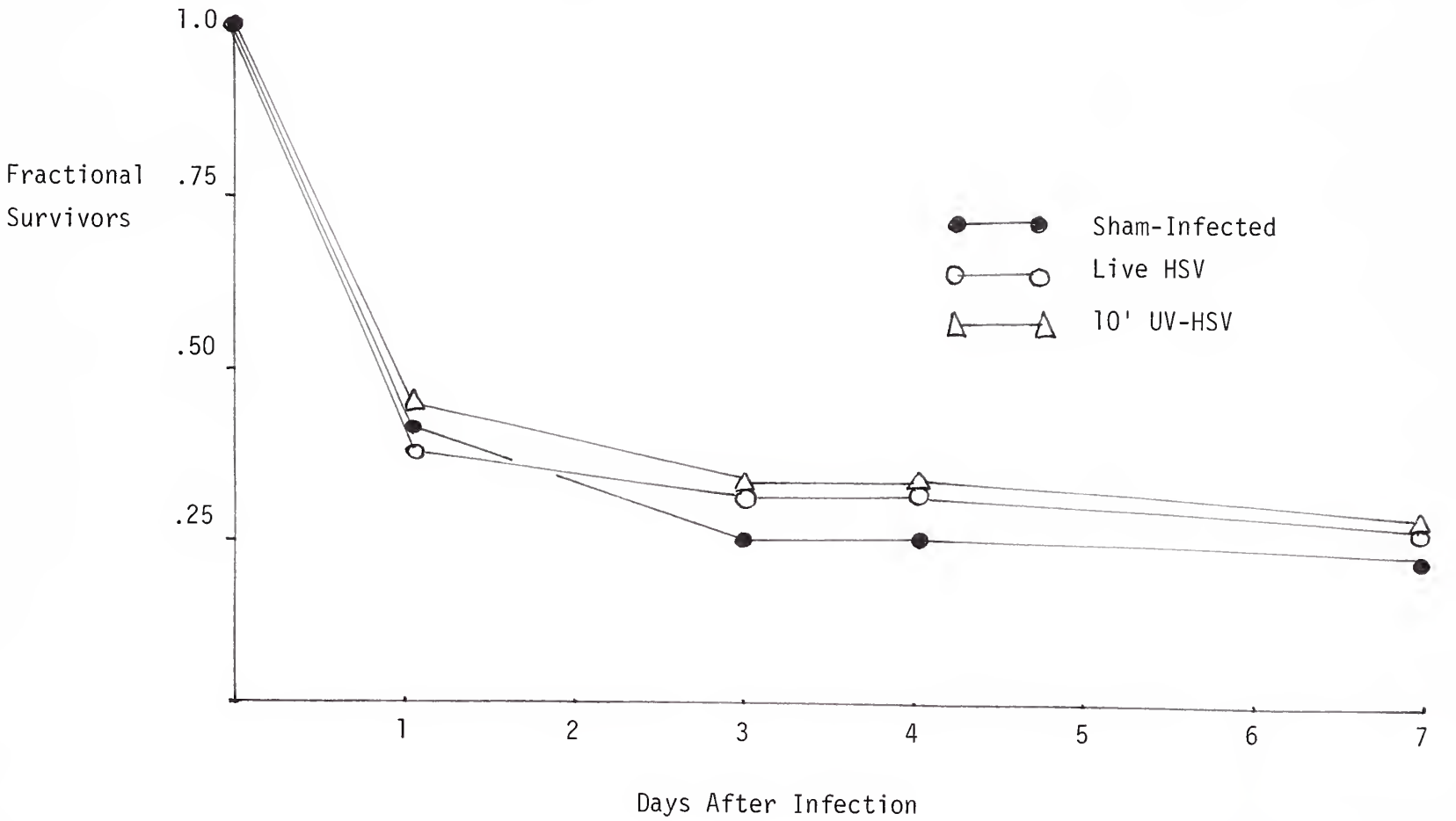
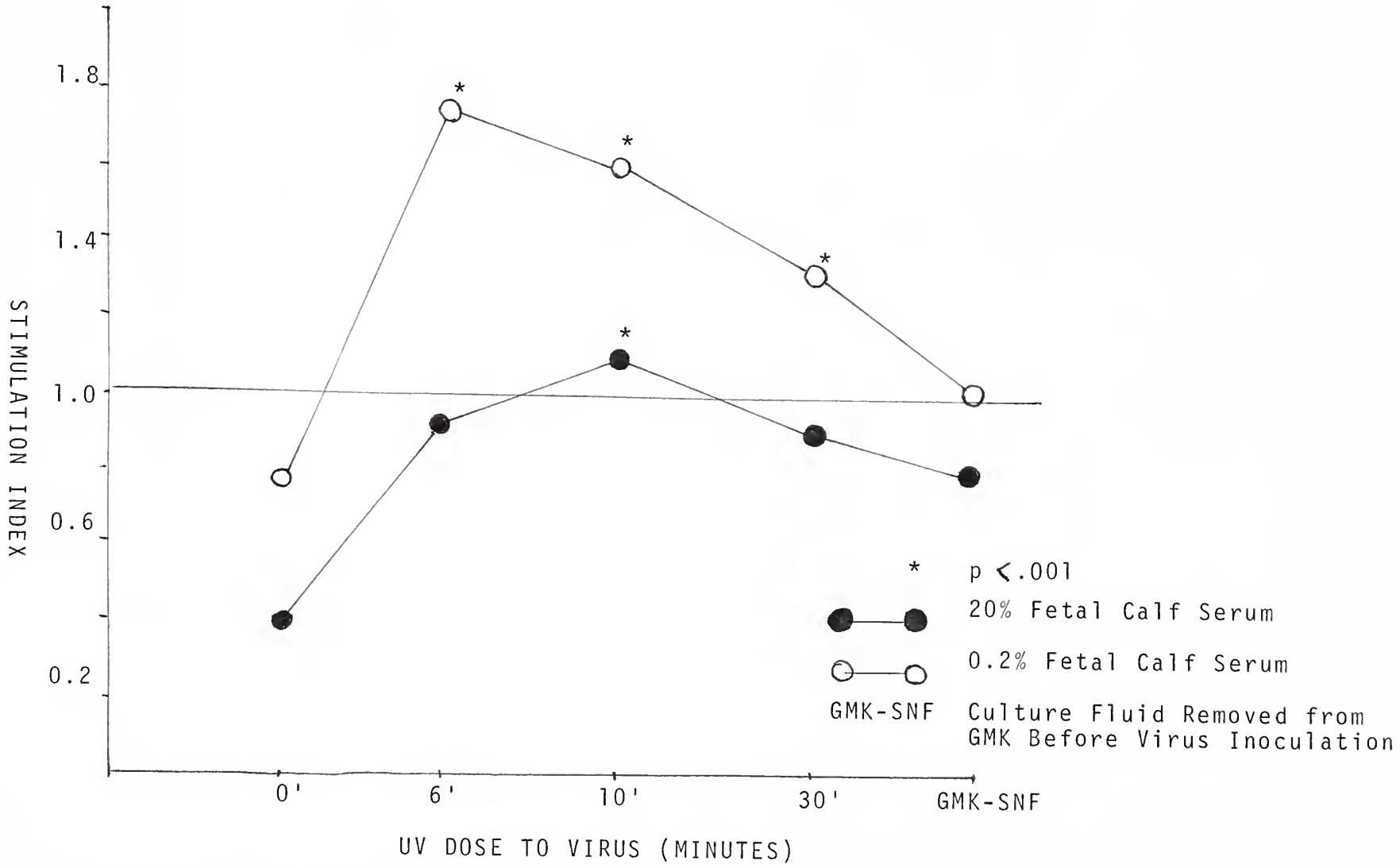


FIGURE 3

LYMPHOCYTE STIMULATION BY UV-HSV



DISCUSSION

Summary. These experiments have shown that live HSV-1 productively infects only a small fraction (1-6/1000) of freshly-isolated human umbilical cord lymphocytes. Maintaining these cells in culture for 5 days or more is associated with a ten-fold increase in productively infected cells. Umbilical cord lymphocytes (HUCL) infected with UV-irradiated HSV exhibit significant increases in thymidine uptake compared with sham-infected controls, after four days in culture. This effect is intensified by the use of reduced serum concentration in the incubation medium and by the enrichment of the cell population for "B" cells. Sucrose gradient purified virus causes the stimulation while unpurified stocks to not; producer-cell (GMK) growth medium and virus suspensions depleted of virus by centrifugation do not cause stimulation; and the virus preparation itself was shown to be free of cellular debris or mycoplasma contamination. Maximal stimulation is caused by virus exposed to ultraviolet light for 6' to 10' and occurs 3 to 4 days after infection.

Association of Lymphocyte Infectivity and Aging in Culture.

The increase in cells susceptible to infection by HSV over

5 to 7 days of *in vitro* culture is similar to the result found in macrophages by Daniels et al.[95] The correlation with the increase in DNA synthesis in cultures over time may suggest that cell cycle and/or metabolism influence susceptibility to HSV infection.[96,97,104] It is also possible that *in vitro* culture selects susceptible cells, and loss of certain cells (e.g. "T" cells) in the first days after isolation inactivates the infection resistance exhibited by the culture when all cell populations are represented in *in vivo* proportions.

Stimulation of Lymphocytes by UV-HSV. A number of uncertainties about the meaning of the demonstrated increase in thymidine uptake persists.

1) Viral Antigen or Genome as Lymphocyte Stimulator? Whether the stimulation is due to antigens on the virus surface or due to the viral genome itself has not been shown. Howver, the use of lymphocytes presumed to be non-immune and washed free of autologous humoral factors suggests that immune mechanisms do not play a part. Starr[109] produced a stimulation index of 25 in adult immune lymphocytes exposed to heat-inactivated HSV antigens but found no stimulation of umbilical cord lymphocytes. The fact that selective UV irradiation of virus, a technique known to function by damaging viral DNA,[70] was required for stimulation in

these experiments further supports the notion that the partially inactivated genome is responsible. If a surface antigen were the stimulatory principle, one might expect increasing stimulation with increasing doses of UV, rather than the convex curve observed (Figure 3).

The possibility that a small number of immunized maternal cells in the umbilical cord blood are responsible for the effect cannot be entirely excluded. Data concerning the extent of maternal cell contamination of human umbilical cord blood is scant. Sharpe[126] examined the metaphases of peripheral lymphocytes in four male neonates and found 0.6% of lymphocytes in one infant had a female karyotype; he concluded that transplacental lymphocyte migration is a rare event.

Fujikura[127] surveyed blood from placentas obtained after delivery for the presence of sickle cells in the offspring of hemoglobin (SS) mothers. He found 8.4% of erythrocytes in the umbilical cord vein were sickled, but Olding[128] found no maternal lymphocytes in fourteen separate samples of umbilical vein blood collected intrapartum. Grogan et al. reported a case of fatal graft-versus-host reaction to lymphocytes of maternal origin in a neonate with severe combined immune deficiency[129] which indicates that although rare, transplacental maternal-fetal lymphocyte transfusion does occur.

Lawler[130] has suggested that the immune-competant fetus may be able to suppress maternal-lymphocyte activation. He demonstrated in mixed maternal-HUCL culture (1:1) that the ratio of mitoses favored fetal cells 6-8:1. Even more convincing is the observation by Olding[131] that 98% of the mitoses in maternal/HUCL cultures stimulated with phytohemaglutinin were in fetal cells. This work was confirmed by Adinolfi.[132]

Thus the possibility that UV-HSV stimulation of umbilical cord lymphocytes is antigenically mediated is remote. The intensification of stimulation in B-enriched and macrophage-depleted cell populations also mitigates against an immune phenomenon: HSV is known to be a "T-dependent" antigen.[114,133] Oppenheim et al. observed that a variety of antigens activated pre-immunized lymphocytes but not umbilical cord lymphocytes; in non-immune cells, only mitogens (PHA, Con A, antilymphocyte globulin) were capable of causing activation.[134]

It is possible that UV-inactivated HSV possesses a mitogen on its surface. Future experiments to determine if stimulation is affected by maternal antibody status or the neutralization of HSV by heat or antiserum will help clarify this issue.

2) Induced Cellular or Viral DNA Synthesis? Uptake

of ^{3}HTdr in UV-HSV exposed cells is considered to be due to increased incorporation of thymidine into DNA and, therefore, increased DNA synthetic activity. Bain reported that uptake of ^{3}HTdr in mixed adult lymphocyte cultures was linear over periods of up to five hours, and that the incorporation was directly into DNA.[135] He documented the formation of numerous degradation products of ^{3}HTdr, but these were not included into acid-insoluble material.

Whether thymidine incorporation is associated with viral or cellular DNA synthesis in these experiments has not been determined. Indirect evidence suggest, however, that UV-HSV probably stimulates cellular DNA synthesis: numerous reports have documented HSV replication in lymphocytes over 3-5 days after infection[97,101,109] but Rinaldo[97] found no increase in ^{3}HTdr uptake in infected cultures versus controls; Yamanishi et al.[66] compared ^{3}HTdr uptake by hamster cells exposed to live and temperature-mutant HSV and observed stimulation only in the mutant-infected cells.

Future experiments should determine, via cesium chloride density gradients, whether the ^{3}HTdr is incorporated into viral or cellular DNA.

3) <u>^{3}HTdr Uptake: DNA Repair or Semi-Conservative Replication?</u>

Thymidine incorporation may be involved in the replication of DNA (mitogenesis) or in the repair of damaged DNA. These experiments have not addressed this issue, but differentiation of these possibilities is important because the introduction of UV-damaged viral DNA into the cell may of itself stimulate endogenous repair mechanisms for correction of defects in the viral genome. Radman[92] has proposed that the introduction of damaged viral DNA may provoke an "error prone" cellular DNA repair system; during such repair, mutagenesis of the cell occurs through incorrectly repaired genome sequences. Future experiments to determine if stimulated cultures are undergoing semi-conservative DNA synthesis or repair should help resolve this important question.

4) ^{3}Htdr Uptake: Increased DNA Synthesis or Increased Thymidine Kinase Activity?

DNA replication in eukaryotic cells involves the incorporation of thymidine (as thymidine triphosphate (TTP)), mainly derived from the conversion of uridine monophosphate via the so-called endogenous pathway.[136] A very small fraction of the TTP is derived from pre-formed thymidine; pre-formed thymidine enters the DNA synthetic cycle via the "salvage" pathway and the enzyme thymidine kinase. Stimulation assays utilizing ^{3}HTdr depend on the salvage pathway to

measure thymidine integration into DNA and thus to infer relative rates of DNA synthesis.

Numerous studies cited above[79,85,103] documented the transfer of the thymidine kinase (TK) gene from the HSV genome to mouse cells lacking that enzyme. It is also possible that importation of the TK gene on HSV during infection of lymphocytes induces increased activity of this enzyme. Increased uptake of ^{3}HTdr could then occur without a net increase in DNA synthesis.

Arguing against this notion is the report by Starr et al. that no increased ^{3}HTdr uptake occurred in HUCL exposed to heat-inactivated HSV[109] although heat-inactivation of the TK gene in that experiment cannot be excluded. Yamanishi reported a net increase in cellular DNA synthesis in hamster cells infected with a termperature-sensitive mutant of HSV.[66] However it is important that future work be able to show that UV-HSV infected cultures exhibit thymidine kinase activity similar to controls and that net DNA synthesis is increased.

5) Effect of Serum Starvation: Reduction of Background or Enhanced Susceptibility to HSV?

Reduction of the serum supplementation in other cell systems has had the effect of reducing the background DNA synthesis

and repair, and presumably unmasking low-level stimulatory effects,[66,74,125,137] but this technique has not been applied to human leukocytes. In the present studies, counts per minute (cpm) of medium-inoculated cultures after four days were 30% lower in 0.2% fetal calf serum (FCS) than in 20% FCS, although in one experiment no difference was noted. By 7 days, activity in the 0.2% FCS cultures was 50% less than that of the 20% FCS. It is likely that the lowered serum concentration "arrested" the cells. Johnson[138] has suggested, however, that fetal calf serum contains a mitogenic substance and Alford et al.[139] reported that lymphocyte transformation by mitogens was more efficient when autologous serum was added than with heterologous or calf serum.

The last two reports indicate that fetal calf serum in the medium may inhibit transformation or at least increase the baseline mitogenesis in the culture. It may be that the resistance of lymphocytes to infection and transformation by HSV is attributable to the metabolic state conferred by the fetal calf serum. Further investigation of the varying effects of fetal calf serum and autologous serum is warranted.

Lymphocytes and HSV: Prospects for the Future.

The basis for the striking resistance of the lymphocyte

to infection and transformation by HSV remains unknown. It is likely that this resistance transcends the immune functions of these cells, for even isolated primary "B" lymphocytes are refractory.

Some of the findings in the present experiments ring true to work done in other cell systems: the optimal stimulation dose of UV (6' to 10') is very similar to the dose used in transforming rodent cells (Table I); the use of serum starvation enhanced detectable stimulation although the cells remained viable.[66,74,125,137] These similarities suggest that certain intrinsic properties of the HSV genome are unmasked by limited UV damage to the DNA, and that damaged virus is capable of interacting with cells, probably all cells, in a characteristic fashion which may, in the right setting, involve oncogenic transformation.

Extension of the present findings will require careful exclusion of the possibilities of induced TK and DNA-repair activity and antigenic interaction with maternal HUCL contaminants, as discussed above. Several virus strains should be screened for stimulatory ability (cf. Rapp and Duff's finding of 2/12 transforming strains[38]). Assays conducted over longer term (21 days) may assist in determining if the stimulation in 0.2% fetal calf serum is transitory or the harbinger of transformation of a small clone of cells. Finally, if dedicated effort to induce

outgrowth of a transformed cell line is successful, these cells should be tested for the presence of HSV antibodies and/or HSV genomes.

Further elucidation of the effects documented in this paper may prove fruitful in demonstrating new aspects of the action of herpesviruses in oncogenesis, and at the very least may shed light on the peculiar ability of the lymphocyte to abort viral infection and replication.

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